JOURNAL OF VIROLOGY, July 1972, p. 153–156
Vol. 10, No. 1

Rescue of Rous Sarcoma Virus from Rous Sarcoma Virus-Transformed Mammalian Cells[1]

JOHN M. COFFIN[2]
McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 27 March 1972

Rat cells transformed by the B77 strain of avian sarcoma virus produce no virus-like particles, yet B77 virus was rescued from these cells by Sendai virus-mediated fusion with chicken cells. This virus rescue was not affected by treatment of the chicken cells with agents that rendered the cells incapable of dividing, although such treatment greatly reduced the ability of the chicken cells to plate as infectious centers after infection with B77 virus. Fusion of R(B77) cells with chicken erythrocytes also led to virus rescue, although with less efficiency than fusion with chicken fibroblasts. Therefore, virus rescue was probably due to a factor or factors contributed by chicken cells which aid in virus production.

Some strains of Rous sarcoma virus (RSV) can infect mammalian cells in vivo and in vitro (1, 16). In general, RSV-infected mammalian cells have transformed properties and contain RSV information (11), but produce no RSV-like particles (5). Infectious RSV, with properties similar to those of the infecting RSV strain (1), can be "rescued" from RSV-infected mammalian cells by cocultivation or fusion with chicken cells (14). The nature of the block in virus production and the mechanism of virus rescue are unknown.

R(B77) cells are a line of rat embryo fibroblasts transformed in vitro by the B77 strain of avian sarcoma virus (1, 9). They have been studied extensively in this laboratory (1–3, 7, 9; Coffin and Temin, J. Virol. **9**:766–775). R(B77) cells produced no B77 virus particles detectable by infectivity, ^{3}H-uridine labeling of cells and examination of supernatant fluids, deoxyribonucleic acid (DNA) polymerase activity in supernatant fluids (3), or early interference with B77 virus (7). They contained DNA hybridizable to RSV ribonucleic acid (RNA; M. A. Baluda, Proc. Nat. Acad. Sci. U.S.A. **69**:766–775, 1972). They also contained approximately 7% as much RNA hybridizable to B77 virus DNA (Coffin and Temin, J. Virol. **9**: 766–775) as uninfected chicken cells. They did not, however, contain detectable avian leukosis virus group-specific antigen (J. M. Coffin, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1972) or precursor particles (3) similar to those found in RSV-infected chicken cells (2).

The experiments presented here were performed to study the rescue of virus from R(B77) cells. The following system was used to assay virus rescue from RSV-infected mammalian cells. Sendai virus (a gift of D. Walker, University of Wisconsin) was grown in 8- to 10-day embryonated eggs, and the choroallantoic fluids were harvested after 3 days. Virus was concentrated by centrifugation, resuspended in one-tenth volume of Eagle's medium, and inactivated by exposure for 10 min to a G15T8 germicidal lamp at a distance of 17 cm. Cell fusion was performed by a modification of the procedure suggested by Davidson (4). Chicken embryo fibroblast cultures were prepared from White Leghorn embryos (Sunnyside Hatchery, Oregon, Wis.) as previously described (15). All embryos used were free of avian leukosis virus capable of interfering with RSV of subgroups A, B, C, or D. One-day-old secondary chicken embryo fibroblast cultures in 60-mm culture dishes were washed twice with 2 ml of cold Eagle's medium, and 0.1 ml containing approximately 1,000 hemagglutinating units of concentrated ultraviolet (UV)-inactivated Sendai virus was added to each culture. The cultures were incubated for 10 min at 4 C to allow attachment of the virus, and were then washed twice more with cold Eagle's medium. R(B77) cells or XC cells (a gift of V. Klement) were treated with 5,000 r of gamma irradiation by exposure at a distance of 11 cm to a 2,000-Ci ^{137}Cs source (Cesatron, Atomic Energy of Canada, Ltd.). Amounts of 0.2 ml of Eagle's medium containing gamma-irradiated R(B77) or XC cells were then added to the Sendai virus-treated chicken cells, and the cultures were incubated at 37 C for 40

[1] This work has been submitted as a portion of a Ph.D. thesis, University of Wisconsin, 1972.

[2] Present address: Institut für Molekularbiologie der Universität Zürich, Hönggerberg, 8049 Zurich, Switzerland.

min. A 3-ml amount of Eagle's medium with 20% tryptose phosphate broth (ET medium) containing 5% calf serum was added to all cultures, and the cultures were incubated overnight at 37 C. The supernatant medium was then removed, and 5 ml of ET medium with 5% calf serum and 0.8% agar was added to all cultures. Foci were counted 7 days after fusion.

This assay had the following properties: within the same experiment, replicate cultures had similar numbers of foci (within experimental error); the number of foci observed after fusion of gamma-irradiated R(B77) cells with chicken cells was directly proportional to the number of R(B77) cells plated; and the efficiency of focus formation by R(B77) cells in such experiments determined by an end point technique, that is, fusing small numbers of gamma-irradiated R(B77) cells with chicken cells and replating those cultures which had no foci (15), was similar to the efficiency calculated from direct focus counting of cultures which had been fused with larger numbers of gamma-irradiated R(B77) cells (data not shown).

The experiment shown in Table 1 was performed to compare the efficiency of rescue of RSV from R(B77) cells, a clone of R(B77) cells, and XC cells. XC cells are a line of cells from a rat tumor induced by Prague-strain RSV (12) which have been frequently used previously in studies of this sort (8). The RSV-infected mammalian cells were treated with gamma irradiation, and various numbers of them were plated on Sendai virus-treated chicken cells as described above. R(B77) cells yielded about 10^{-2} to 10^{-3} foci per cell by this procedure. R(B77) clone D6 cells were about twice as efficient at producing foci as uncloned R(B77) cells, and XC cells were about one-tenth as efficient at producing foci as R(B77) cells.

In numeruos experiments of this sort, the efficiency of focus formation (in foci per cell) of gamma-irradiated R(B77) cells fused with chicken cells varied between approximately 10^{-2} to 10^{-3}. The foci observed after fusion of gamma-irradiated R(B77) cells with chicken cells were similar in appearance to foci induced by B77 virus, consisting of compact clusters of round cells. Thus, the system described here provided a convenient and reproducible means of assaying virus rescue from RSV-transformed mammalian cells.

A number of models for rescue of RSV from RSV-transformed mammalian cells have been proposed (13). These include: (i) infection of chicken cells by a labile viral or subviral particle released from the mammalian cells aided by close contact between the two cell types and by the presence of Sendai virus; (ii) "infection" of chicken cells by an intracellular subviral particle from the mammalian cells, which requires heterokaryon formation for transfer to the chicken cells; (iii) addition to the mammalian cell-chicken cell heterokaryons of some factor in chicken cells required for production of infectious RSV; and (iv) dilution of a repressor in the mammalian cells. The following experiments were performed to distinguish between these models.

Under models i and ii, above, initiation of virus production after fusion of chicken cells with RSV-transformed mammalian cells should have properties similar to initiation of virus production after infection of chicken cells by RSV. One of the properties of infection of chicken cells by RSV is its sensitivity to pretreatment of the cells by X irradiation or other agents, such as mitomycin C, which reduce the ability of the cells to divide (10). To determine whether rescue of virus from R(B77) cells by fusion with chicken cells shared this prop-

TABLE 1. *Recovery of RSV after fusion of cloned and uncloned R(B77) cells and XC cells with chicken cells*[a]

Expt[b]	Cell type[c]	Sendai virus[d]	No. of cells plated	Foci[e]	Foci per cell
A	R(B77) uncloned	+	10^4	32, 27	3.0×10^{-3}
		−	10^5	0, 0	$<5 \times 10^{-6}$
	XC	+	10^5	8, 17	1.3×10^{-4}
		−	10^5	0, 0	$<5 \times 10^{-6}$
B	R(B77) uncloned	+	10^3	16, 7	1.2×10^{-2}
	R(B77) clone D6	+	10^3	25, 23	2.4×10^{-2}

[a] Cells were fused with approximately 5×10^5 chicken embryo fibroblasts as described in Materials and Methods, and were overlaid with ET medium with 0.8% agar and 5% calf serum the next day.
[b] The experiments were performed at different times by the same procedure.
[c] All cells were irradiated with 5,000 r of gamma irradiation.
[d] Approximately 1,000 hemagglutinating units of UV-inactivated Sendai virus.
[e] Foci were counted 7 days after fusion.

erty with infection of chicken cells by RSV, the following experiment was carried out (Table 2). Cultures of secondary chicken, mouse, or rat cells were treated with 5,000 r of gamma irradiation or with 10 μg of mitomycin C per ml (Calbiochem, Los Angeles, Calif.) for 2 hr, or were left untreated. The mitomycin C treatment was sufficient to reduce incorporation of ^{3}H-thymidine into DNA between 1 and 2 days after treatment to less than 5% of that of control cultures (data not shown). All cultures were treated with UV-inactivated Sendai virus, and various amounts of gamma-irradiated R(B77) cells or of B77 virus were added. The next day, 5×10^5 untreated chicken cells were added to all cultures. Foci were counted 7 days later. Pretreatment of chicken cells by gamma irradiation or mitomycin C greatly reduced the ability of B77 virus-infected chicken cells to act as infectious centers. Focus formation by gamma-irradiated R(B77) cells fused with chicken cells was not significantly reduced by pretreatment of the chicken cells with mitomycin C or gamma irradiation. To determine whether the focus formation observed when R(B77) cells were fused with mitomycin C-treated or gamma-irradiated chicken cells was due to Sendai virus remaining in the cultures after 1 day, R(B77) cells were fused with irradiated rat cells or mitomycin C-treated mouse cells and chicken cells were added the next day. These latter fusions produced less than 1% as many foci per cell as did fusion with gamma-irradiated or mitomycin C-treated chicken cells. Therefore, most of the foci observed after fusion of R(B77) cells with killed chicken cells were not due to spontaneous fusion of the R(B77) cells with the added untreated chicken cells, or to residual Sendai virus. Thus, rescue of RSV from RSV-transformed mammalian cells after fusion of chicken cells can occur even when both cell types are rendered incapable of dividing. These results indicate that rescue of RSV from RSV-transformed mammalian cells does not occur by a mechanism that requires cell cycle-dependent activation, as does infection with RSV (15). Models i and ii above are, therefore, ruled out. This conclusion is supported by the results of Machala et al., who observed that shortly after fusion of XV cells with chicken cells all heterokaryons contained greater amounts of RSV group-specific antigens than did XC cells alone (8).

A contradictory result has been reported by Jonsson (6). He did not detect virus in the supernatant fluids after fusion of RSV-infected mammalian cells and irradiated chicken cells. In the course of the experiments described here, significant infectious virus released into the supernatant medium after fusion of R(B77) cells and gamma-irradiated chicken cells was not detected. It is likely in such experiments that such a small quantity of virus is released by heterokaryons that only a sensitive infectious center assay like that employed here can detect it.

The conclusion that virus rescue is not due to "infection" of chicken cells is supported by the following experiment (Table 3). Gamma-irradi-

TABLE 2. *Comparison of the ability of gamma-irradiated or mitomycin C-killed chicken cells to act as infectious centers after fusion with R(B77) cells or after infection with B77 virus*[a]

Expt	Cell type	Fused with R(B77) cells[b]		Infected with B77 virus	
		Efficiency[c]	Efficiency relative to untreated chicken cells	Apparent titer[d]	Titer relative to untreated chicken cells
A	Untreated chicken	7.6×10^{-4}	1.0	2.9×10^5	1.0
	Irradiated chicken[e]	4.2×10^{-4}	0.6	1.1×10^4	0.04
	Irradiated rat[e]	5.0×10^{-6}	0.007	7.0×10^3	0.02
B	Untreated chicken	2.1×10^{-3}	1.0	3.2×10^6	1.0
	MC-chicken[f]	3.3×10^{-3}	1.6	3.7×10^4	0.01
	MC-mouse[f]	5.0×10^{-6}	0.002	8.5×10^3	0.003

[a] Cultures of the indicated cell type were treated with approximately 1,000 hemagglutinating units of UV-inactivated Sendai virus and were overlaid with various amounts of R(B77) cells or B77 virus. The next day, 5×10^5 untreated chicken cells were added, and an agar overlay was added 1 day later. Foci were counted 7 days after the overlay was added.

[b] The R(B77) cells were treated with 5,000 r of gamma irradiation immediately prior to fusion.

[c] Foci per R(B77) cell. Each number represents the average of determinations on two cultures.

[d] Focus-forming units per milliliter.

[e] Treated with 5,000 r of gamma irradiation 3 days before fusion.

[f] Treated with 10 μg of mitomycin C per ml for 2 hr, 2 days before fusion.

TABLE 3. *Comparison of the ability of chicken embryo fibroblasts and chicken embryo erythrocytes to rescue virus from R(B77) cells*[a]

Cell type	No. of R(B77) cells plated	No. of foci[b]	Foci per cell
Chicken embryo fibroblast[c]	10^5	180	1.8×10^{-3}
Chicken embryo erythrocyte[d]	5×10^5	25	5.0×10^{-5}
None	5×10^5	0.5	1.0×10^{-6}

[a] Cultures containing approximately 5×10^6 R(B77) cells were irradiated with 5,000 r of gamma irradiation, treated with approximately 1,000 hemagglutinating units of UV-inactivated Sendai virus, and overlaid with the indicated cell type. The next day, the indicated numbers of the R(B77) cells were replated on chicken embryo fibroblast cultures. An agar overlay was added the following day, and foci were counted 7 days later.

[b] Average of six replicate cultures.

[c] The number of cells per R(B77) culture was 1.1×10^6.

[d] The number of cells per R(B77) culture was 7.2×10^6.

ated R(B77) cells were treated with Sendai virus, and chicken embryo erythrocytes [approximately 1 per R(B77) cell], fibroblasts [approximately 0.2 per R(B77) cell], or no cells were added. The next day, all cultures were replated as infectious centers on secondary chicken cell cultures. Foci were counted 7 days later. The number of foci observed when R(B77) cells were fused with chicken erythrocytes was approximately 50-fold greater per R(B77) cell than the number of foci in the control cultures. About 40-fold fewer foci per cell were observed when the R(B77) cells were fused with chicken embryo erythrocytes than blasts. This lower efficiency may have been related to a lower efficiency of heterokaryon formation between R(B77) cells and erythrocytes. Thus, cells which are naturally incapable of dividing can aid in the rescue of RSV from RSV-transformed mammalian cells. Neutralization of a repressor in R(B77) cells by a factor in chicken cells is possible.

Rescue of virus from RSV-infected mammalian cells, then, occurs either by dilution of a repressor, or by addition of some factor to the cells which is necessary for virus production. It is unlikely that a repressor is involved in inhibiting virus production by R(B77) cells for two reasons. For one, there is no reason to expect the presence of a repressor acting on RSV in mammalian cells or coded by the RSV genome. In addition, the dilution of repressor on fusion of an R(B77) cell with a chicken embryo fibroblast, and especially with an erythrocyte, is not likely to be sufficient to cause derepression in heterokaryons.

It is, therefore, likely that the chicken cell provides some factor or factors required for the production of RSV to R(B77) cell-chicken cell heterokaryon. This factor can be supplied by chicken embryo erythrocytes, although with less efficiency than by fibroblasts.

I thank Howard M. Temin for his guidance and support of this work, and R. Chen and A. Kato for technical assistance.

This investigation was supported by Public Health Service research grant CA-07175 from the National Cancer Institute. I was supported by training grant T01-CA-5002 from the National Cancer Institute.

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